Session 4: Calcium signaling

Lectures

L4.1

Regulation and physiological function of store-operated calcium entry

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Activation of phospholipase C results in release of intracellular Ca^{2+} and activation of Ca^{2+} entry. Plasma membrane Ca^{2+} entry most commonly is signaled by the depletion of intracellular Ca^{2+} stores, a mechanism referred to as *capacitative calcium entry* or *store-operated calcium entry*. Recent work from a number of laboratories has highlighted the roles of Ca^{2+} sensor proteins, STIM1 and 2, and a channel subunit, Orai1. In addition to Orai1, two other related mammalian proteins, Orai2 and 3, are capable of forming store-operated channels with pore properties similar to Orai1. STIM1 activates Orai channels by a mechanism that depends upon its co-localization with Orai at endoplasmic reticulum – plasma membrane junctions. STIM1 is organized within the endoplasmic reticulum by mechanisms dependent on the microtubular cytoskeleton and collects upon store depletion at specific sites. STIM1 plays an important role during calcium oscillations, not only providing calcium to maintain intracellular stores, but as an important route for calcium signaling to downstream effectors. During mitosis, store-operated entry is down-regulated by complex mechanisms involving changes in protein expression as well as co-valent modification of STIM1 by phosphorylation of multiple sites in a C-terminal regulatory domain.

L4.2

Calcium signalling during excitation-contraction coupling in atrial myocytes

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Electrical depolarisation of ventricular myocytes produces homogenous, global increases in calcium to trigger contraction during each heart beat. Such global calcium transients arise due to the simultaneous recruitment of many calcium spark sites throughout each cell. An essential component of ventricular myocytes is their 'transverse tubule system' (T-tubules), which conveys the action potential deep into the cells to ensure that calcium sparks are simultaneously triggered throughout the volume of a myocyte. In many species, atrial myocytes do not express T-tubules, and consequently their calcium signals are spatially distinct [1]. Atrial myocyte calcium transients originate in sub-sarcolemmal locations, and give rise to a sharply-defined ring of elevated calcium around their periphery. Positive inotropic agents induce globalisation of action potential-evoked calcium signals in atrial myocytes, and correspondingly a significant increase in the contractility of the cells, by promoting the centripetal, inward propagation of the calcium transients so that calcium can reach the contractile filaments [2]. From the description given above, it could appear that atrial myocytes are simply ventricular myocytes without T-tubules. We compared calcium signalling in atrial myocytes and chemically de-tubulated ventricular myocytes. Our findings indicate that atrial myocytes have unique calcium signalling kinetics that are not mimicked by simply removing the T-tubules from ventricular myocytes [3]. We have developed a mathematical model of atrial myocyte calcium signalling based on the realistic distribution of calcium spark sites (ryanodine receptor clusters). Our model recapitulates the sub-sarcolemmal initiation of calcium signals in atrial myocytes, and their subsequent centripetal propagation as calcium waves. Furthermore, the model allows predictive analysis concerning the movement of calcium within/between z-disks, the potentially pro-arrythmic effects of altering the refractory period of calcium spark sites or introducing randomness in calcium release thresholds.

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L4.3

The BH4 domains of Bcl-2 and Bcl-Xl differentially regulate inositol 1,4,5-trisphosphate receptors and apoptosis

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Anti-apoptotic Bcl-2 and Bcl-Xl modulate intracellular Ca^{2+} signals and therefore cell fate. We recently found that Bcl-2 directly interacts *via* its BH4 domain with the central, modulatory domain 3 (a.a. 923-1581) of the inositol 1,4,5-trisphosphate receptor (IP_3R), thereby suppressing pro-apoptotic Ca^{2+} signals and protecting cells against apoptotic stimuli. Although Bcl-2 and Bcl-Xl show high similarities in sequence and structure, they seem to differentially regulate IP_3Rs. Therefore, we compared the role of the BH4 domains of Bcl-2 and Bcl-Xl in the regulation of IP_3R-dependent Ca^{2+} signaling and apoptosis.
First, in GST pull-down and surface plasmon resonance experiments, we found that the BH4 domain of Bcl-2 strongly interacted with domain 3 of the IP_3R (K_d: ~1mM), whereas that of Bcl-Xl displayed a much weaker interaction. Both BH4 domains did not significantly interacted with the C-terminal tail (domain 6: a.a. 2590-2749) of the IP_3R. Second, unidirectional $^{45}Ca^{2+}$ fluxes in permeabilized cells revealed that the BH4 domain of Bcl-2 potently inhibited IP_3-induced Ca^{2+} release (IICR) (IC_{50}: ~35 mM), whereas the BH4 domain of Bcl-Xl did not. Third, we used a FITC-VAD-FMK marker to determine the apoptotic index (AI) of cells co-electroporated with purified cytochrome C (CytC) and Bcl-2- or Bcl-Xl-BH4 peptides. Again, the BH4 domain of Bcl-2 was much more effective (AI: ~40% of control) than that of Bcl-Xl (AI: ~70% of control) in protecting cells against CytC-induced apoptosis. Furthermore, the protective effect of the BH4 domain of Bcl-2 was completely abolished (AI: ~ 95% of control) by co-electroporation of a competing peptide corresponding to the Bcl-2-binding site of the IP_3R (a.a. 1389-1408), whereas this peptide did not affect the protective action of the BH4 domain of Bcl-Xl. Fourth, we mapped the residues responsible for the inhibitory effects of the BH4 domain of Bcl-2 and found 3 amino acids (Lys17, Tyr21 and Arg26) to be essential. Importantly, Lys17 was not conserved in Bcl-Xl and corresponded to an aspartate residue (Asp11) in the latter. Strikingly, changing Lys into Asp in the BH4 domain of Bcl-2 was sufficient to abolish its inhibitory effects on IICR, whereas changing Asp into Lys in the BH4 domain of Bcl-Xl provoked inhibition of IICR.
We conclude that the BH4 domains of Bcl-2 and Bcl-Xl although are very similar in sequence and structure, differentially affect IP_3R function and inhibit apoptosis by divergent mechanisms.

L4.4

NAADP regulates human platelet function

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Platelets play a vital role in maintaining haemostasis. Human platelet activation depends on Ca^{2+} release, leading to cell activation, granule secretion and aggregation. NAADP is a Ca^{2+}-releasing second messenger, with actions demonstrated in a number of mammalian systems. It acts to release Ca^{2+} from acidic stores, rather than the endoplasmic reticulum. The NAADP-sensitive Ca^{2+} channel has recently been identified as the two pore channel. NAADP can induce Ca^{2+} release in permeabilised human platelets and is thought to contribute to thrombin-mediated platelet activation. Here we characterise NAADP-mediated Ca^{2+} release in human platelets. Using a radioligand binding assay, we reveal an NAADP binding site in human platelets, strongly suggesting the presence of an NAADP receptor. Ned-19, a novel cell-permeant NAADP receptor antagonist, competes for this binding site. NAADP is demonstrated to release Ca^{2+} from platelets in both Mn^{2+} quench and $^{45}Ca^{2+}$ assays. The store from which NAADP releases has been investigated using pharmacological approaches. Ned-19 inhibits both thrombin and CRP-induced Ca^{2+} release in human platelets and has an inhibitory effect on platelet aggregation and granule secretion. In addition, we have measured changes in NAADP levels in response to activation with both thrombin and CRP. We conclude that NAADP plays a role in human platelet function. Furthermore, Ned 19 provides a potential avenue for platelet-targeted therapy and the regulation of thrombosis.

L4.5

Recruitment of annexin A11 to endoplasmic reticulum exit sites is mediated by the adaptor function of the penta-EF-hand protein ALG-2

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Annexin A11 is a member of a large family of Ca^{2+}-dependent phospholipid-binding proteins with roles in Ca^{2+} signaling, apoptosis, and vesicle trafficking. We previously reported that the penta-EF-hand protein ALG-2 Ca^{2+}-dependently binds the N-terminus of annexin A11. ALG-2 also interacts with a COPII component Sec31A and localizes at endoplasmic reticulum exit site (ERES). Since ALG-2 forms homodimers that could cross-bridge two molecules, it is possible that ALG-2 recruits its interacting proteins to the ERES. In this presentation, we first demonstrated that annexin A11 was Ca^{2+}-dependently recovered in the pull-down product of Strep-tagged Sec31A, but not in that of the mutant lacking the ALG-2 binding site. Second, both Sec31A and ALG-2 were detected in immunoprecipitates of anti-annexin A11 from the lysate of HEK293 cells in the presence of Ca^{2+}. The association between annexin A11 and Sec31A required ALG-2 because of the absence of Sec31A in the anti-annexin A11 immunoprecipitates from the ALG-2-depleted cells by RNAi technology. The association was restored by overexpression of the RNAi-resistant form of ALG-2, but not a shorter isoform of ALG-2 that does not bind annexin A11. Third, indirect immunofluorescence analysis revealed that endogenous annexin A11 of HT1080 cells predominantly located in the nucleus but also distributed in the cytoplasm in a punctate pattern. Some of the puncta were labeled with anti-Sec31A and anti-ALG-2 antibodies. Treatment of cells with brefeldin A or nocodazole resulted in a concentration of annexin A11 in the Sec31A-positive structures. Finally, the recruitment of annexin A11 to the Sec31A-positive compartments was confirmed by live-cell imaging analyses, in which green fluorescent protein-fused annexin A11 (AnxA11-GFP) and red fluorescent protein-fused Sec31A (Sec31A-RFP) were expressed in HeLa cells. After histamine administration, cytoplasmic AnxA11-GFP transiently accumulated at Sec31A-RFP-positive puncta. The redistribution of AnxA11-GFP after histamine stimulation was not seen in ALG-2-knockdown cells and exogenous expression of ALG-2 in the ALG-2 knockdown cell resulted in restoration of the AnxA11-GFP redistribution in response to Ca^{2+}-mobilization. These results demonstrate the recruitment of annexin A11 to Sec31A-positive ERES by the adaptor function of ALG-2.

Posters

P4.1

The endoplasmic reticulum-Ca^{2+} store of HEK293 cells is not homogeneous

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The release of Ca^{2+} from intracellular stores plays a crucial role in controlling a whole array of cell functions. Previous studies have indicated that the intracellular Ca^{2+} pool may be heterogeneous and exhibit different properties in accumulating and releasing Ca^{2+}. Here we have characterized the intracellular stores of HEK293 and HeLa cells by following $[Ca^{2+}]$ changes 1)in the cytosol, measured with Fura-2, and 2)in the endoplasmic reticulum (ER) with ER-targeted aequorin. Both HEK293 and HeLa cells take up Ca^{2+} into the ER to near 10^{-3} M levels. This Ca^{2+} pool is mobilizable by cell stimulation with IP_3-producing agonists such as carbachol or ATP. The sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor thapsigargin prevents refilling of >90% of this pool, as reported by ER-targeted aequorin consumption. Interestingly, we have identified in HEK293 but not in HeLa cells, a distinct Ca^{2+} pool insensitive to the SERCA inhibitor, 2,5-di(tert-butyl)-1,4-benzohydroquinone (TBH), and releasable by maximal stimulation with carbachol plus ATP. This store is neither basic, since it is not inhibited with propionic acid, nor acidic, since it is not collapsed by the weak base trimethylamine. Moreover, nigericin was unable to block the TBH-resistant cytosolic increase. Finally, treatment with GPN (Gly-Phe-β-Naphtylamide), a lysosomal disruptor, did not inhibit the peak, indicating that this Ca^{2+} store has no lysosomal characteristics.

Direct measurements of the $[Ca^{2+}]_{ER}$ with targeted aequorins showed that the ER of HeLa cells was unable to refill in the presence of TBH, whereas the one of HEK293 cells refilled up to a 25% of the control without TBH. Furthermore, ATP plus carbachol completely emptied this Ca^{2+} store, indicating that the TBH-resistant pool is indeed a part of the ER. Measurements of Ca^{2+}-uptake in digitonin-permeabilized cells suggests that the Ca^{2+} affinity of the TBH-resistant uptake mechanism is lower than the one responsible for the bulk Ca^{2+} uptake.

We have checked the expression of different isoforms of SERCA3 by Q-RT-PCR, confirming that SERCA 3d is more abundant in HEK cells than in HeLa cells. Importantly, ATPase activity is fully prevented by TBH in HeLa but not in HEK cells. Finally, HeLa cells overexpressing SERCA3d mimicked the behaviour of HEK293 cells and showed now a TBH-resistant ER refilling which was releasable by ATP and carbachol. This release was also evidenced by a concomitant cytosolic Ca^{2+} increase.

P4.2

Disruption of the PMCA-calcineurin interaction enhances paclitaxel cytotoxicity in breast cancer cells

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Paclitaxel (Taxol®) is one of the leading drugs currently in use for the treatment of breast cancer. Unfortunately, clinical use of the drug is associated with severe side effects in the patient.Paclitaxel-induced apoptosis of breast cancer cells requires activation of the calcineurin/NFAT signal transduction pathway. We have recently reported that the activity of calcineurin is regulated in breast cancer cells *via* interaction with the plasma membrane protein PMCA2. Interaction of calcineurin with PMCA2 results in inhibition of the calcineurin/NFAT pathway. Conversely, disruption of this interaction leads to an increase in the activity of this pathway. These results prompted us to investigate if the disruption of the interaction PMCA-calcineurin might result in an increase of calcineurin activity that would lead to apoptosis of breast cancer cells.We show here that disruption of the interaction between PMCA and calcineurin significantly reduces the viability of MCF-7, MDA-MB-231, T47D, and ZR-75-1 breast cancer cell lines. Loss of viability is accompanied by up-regulation of the levels of the pro-apoptotic molecule FasL, suggesting that the reduction of cell viability is the result of an increase in apoptosis. Moreover, disruption of the PMCA/calcineurin interaction in the presence of paclitaxel significantly increases paclitaxel-mediated cytotoxicity of breast cancer cells.In conclusion, disruption of the PMCA/calcineurin interaction induces breast cancer cell death and enhances the cytotoxic effect of paclitaxel. These results indicate that this novel molecular strategy might have important therapeutic applications in the future to reduce the doses of paclitaxel being required to kill the breast tumor, and, therefore, to reduce the systemic toxicity of chemotherapy treatment in breast cancer patients.

P4.3

Calcium signalling in rat glioma C6 cells evoked by 2', 3'-O-(4-benzoylbenzoyl)-ATP. Role of the $P2Y_2$ nucleotide receptors

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2',3'-O-(4-benzoylbenzoyl)-ATP (BzATP) is commonly used as agonist of ionotropic, nucleotide $P2X_7$ receptors. Here we show the independence of calcium signalling from the $P2X_7$ receptor activity in glioma C6 cells. In the absence of extracellular Ca^{2+}, BzATP generates increase in $[Ca^{2+}]_i$ *via* Ca^{2+} release from intracellular stores. In the presence of calcium ions BzATP establishes biphasic Ca^{2+} response, in the manner typical for P2Y receptors. Brillant Blue G (BBG), a selective antagonist of the rat $P2X_7$ receptor, does not reduce any of two components of Ca^{2+} response elicited by BzATP. Periodate-oxidized ATP (Ox-ATP) blocks not only BzATP- but also UTP-induced Ca^{2+} elevation. Moreover, BzATP does not open large transmembrane pores. We also observe cross-desensitization between UTP and BzATP, what clearly shows that in glioma C6 cells BzATP activates most likely $P2Y_2$ but not $P2X_7$ receptors.

P4.4

SERCA2a handles agonist-induced intracellular Ca^{2+} signal, controlling transcription factor NFAT and proliferation in human vascular smooth muscle cells

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Excessive proliferation of vascular smooth muscle cells (VSMC) is the primary cause of several vascular diseases. Store-Operated Calcium Entry (SOCE) controls this proliferation through activation of transcription factor NFAT. Here we have investigated the role of cardiac isoform of the SR/ER calcium ATPase (SERCA2a) in the control of SOCE in human coronary artery VSMC (huCASMC).

We observed that SERCA2a expression was reduced in the subendothelial SMC in human coronary arteries and in cultured proliferating huCASMCs. Accordingly, adenovirus-directed SERCA2a gene transfer inhibited NFAT dependent proliferation and migration in huCASMCs. This inhibition is associated to a decrease of both spontaneous basal Ca^{2+} current observed by single-channel patch-clamp recording and spontaneous interactions between STIM1 (SOCE calcium sensor) with Orai2 (SOCE pore forming unit). Finally, calcium imaging revealed that responses of SERCA2a-expressing cells to the physiological agonist thrombin differed from those of control proliferating cells. The latter showed a steady-state increase in cytosolic Ca^{2+} followed by SOCE in cells, whereas in the presence of SERCA2a, thrombin induced rapid Ca^{2+} oscillations without SOCE.

SERCA2a handles intracellular Ca^{2+} signaling from amplitude to frequency modulation, thereby controlling SOCE and NFAT.

P4.5

Involvement of $iPLA_2$ in NOX2 activation through the regulation of p38 MAPK and store-operated Ca^{2+} entry

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Inflammatory cells, in particular neutrophils, are the pivot of the cell-mediated innate immunity responsible for the killing of invading pathogens. Their capacity to release a large amount of reactive oxygen species (ROS) produced by NADPH oxidase (NOX2) is critical for full effectiveness of the bacterial killing function. On the other hand, neutrophils can accumulate in tissues and become inappropriately activated to secrete their cytotoxic products contributing to host tissue damage. As a consequence, an overactivity of neutrophils is associated to a number of human diseases such as rheumatoid arthritis, adult respiratory distress syndrome or Crohn's disease.

Although it is now largely admitted that store-operated Ca^{2+} entry (SOCE) controlled by STIM1 and SOC channels contributed tightly to NOX2 activation [1, 2], other molecular players for SOCE appear capable of regulating NOX2 activity.

Considering the fact that $iPLA_2$ has been found to have a probable role in SOCE [3], we first examined whether this phospholipase arbitrate the regulation of NOX2 activity in neutrophil-like HL-60 cells. Using siRNA and pharmalogical approaches, we showed that down-regulation of $iPLA_2$ activity led to a decrease of extracellular Ca^{2+} entry induced by fMLF or thapsigagin. In addition, fMLF- but not PMA-induced H_2O_2 production was blocked by BEL, an $iPLA_2$ inhibitor, and siRNA targeting $iPLA_2$. On the whole, our results underline the importance of $iPLA_2$ in SOCE-regulated NOX2 activity.

Since it is known, on the one hand, that p38 MAPK participates to NOX2 activation [4] and on the other hand that p38 MAPK and $iPLA_2$ are involved in the same intracellular signaling pathway [5], we thus determined the role of p38 MAPK in $iPLA_2$-regulated NOX2 activation. Cell pretreatment with BEL triggered a decrease in fMLF-induced p38 MAPK activity indicating that $iPLA_2$ acts upstream of p38 MAPK.

In conclusion, this study underlines the fact that $iPLA_2$ regulates NOX2 activity *via* p38 MAPK and SOCE mechanism.

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P4.6

Structural changes in human S100A1 protein induced by Cys85Met mutation

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S100A1 is a homodimeric calcium binding protein containing 93 residues per subunit. It is stabilized by noncovalent interactions at its dimer interface; each subunit contains two EF-hand motifs linked by a flexible linker [1].
Chemical modification of Cys 85 residue in the C-terminal part of S100A1 protein by disulfide bond formation with small thiols such as glutatione, cysteine, or β-mercaptoetanol (βME) leads to a dramatic increase of the protein affinity for calcium [2] due to conformational changes induced in the *apo* protein, most probably, by an interaction of a thiol molecule attached to Cys 85 with aromatic rings of Phe 88 and 89 which keeps the 85-89 segment of the protein in the α-helical conformation [3].
If so, the substitution of Cys 85 by Met, with the side chain long enough to reach Phe 88 and Phe 89 residues in the α-helix should have the similar effect on protein structure and its calcium-binding ability as Cys 85 thionylation.
Possible structural changes in proteins can be detected using chemical shift data. Conformational differences between two proteins usually give rise to substantial changes chemical shifts for given residue [4]. On the other hand, secondary structure elements can be predicted via the evaluation of backbone torsion angles using the TALOS software [5] which is essentially based on the backbone chemical shifts as well.
Using double labeled $^{15}N/^{13}C$ S100A1 and S100A1(C85M) proteins we have determined the chemical shifts of almost all backbone chemical shifts in both proteins. Chemical shift changes closely remind those induced by protein thionylation with β-mercaptoetanol [3]. There are three parts of the protein the most influenced by the mutation: a large part of N-terminal helix I and a part of the adjoined binding loop, the linker, and the vicinity of the mutation site.
Concluding, analysis of the backbone chemical shifts confirms that conformational changes due to mutation at Cys 85 site are similar to those caused by its thionylation. It seems, therefore, that Cys85Met mutation locks permanently S100A1 protein in the structure that enhances its affinity for calcium ions.

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P4.7

Mitosis and cell cycle progression depend on Orai1 and Orai3 expression in HEK293 cells

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Calcium influx is needed for cell proliferation and recent identification of Orai1 and Orai3 as the main constituents for both store-operated (SOCE) and non capacitative (NCCE) calcium entries led us to investigate the role of these two proteins in mitosis and cell cycle progression in HEK293 cells. Cell cycle analysis, calcium entry, and cell cycle progression over a period of 15 hours, were monitored in control and in cells knock downed for Orai1 (siOrai1), Orai3 (siOrai3), and Orai1 and Orai3 (siOrai1+3). Experiments were performed 48 to 72 hours after transfection. 10μM RO-3306, a cyclin dependent kinase 1 (cdk-1) inhibitor was applied for 24 hours to block 90% of HEK293 cells in G2/M phase. Distribution in cell cycle and cell cycle progression after release from RO-3306 block were assessed using FACS analysis. Calcium imaging and whole-cell voltage clamped recordings showed a 70% reduction in SOCE in siOrai1 or siOrai3 transfected cells. Cell cycle analysis showed an increase in G2/M phase and a decrease in S phase in siOrai3 and siOrai1+3 treated cells. The number of cells in G1 and the number of cells in S phase 4 hours and 15 hours after release from block respectively, were twice lower in the absence of one or both calcium channels. Cell cycle progression through mitosis and through G1 phase was slower in the absence of Orai1 and Orai3 than in control cells. Exit from G2/M phase monitored 15 hours after block release was faster in siOrai1 than in siOrai3 or siOrai1+3 cells, but in all cases slower than in control cells. Our results indicated that, although at a slower rate, mitosis and cell cycle progression occur even at low Orai1 and Orai3 expression and little calcium influx.

P4.8

Sorcin is overexpressed in the brain and participates in the regulation of neuronal function: calcium-mediated sorcin network(s) of interactions

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Sorcin is a penta-EF Ca^{2+}-binding protein. Upon Ca^{2+} binding, sorcin undergoes a conformational change which causes exposure of hydrophobic surfaces and translocation from cytosol to membrane, where it interacts with and regulates several target proteins.

In the heart, sorcin participates in the regulation of the excitation-contraction-relaxation cycle. Following depolarization events, Ca^{2+} enters the cell through the depolarization-activated L-type Voltage-dep. Ca^{2+} channels and the Na^{+}-Ca^{2+} exchangers (NCX); Ca^{2+} entry triggers Ca^{2+} release from the sarcoplasmic reticulum (SR) channels, in particular the ryanodine receptor (RyR). Ca^{2+} release from SR raises cytosolic [Ca^{2+}], allowing Ca^{2+} to bind to troponin C, which switches on the contractile machinery. Following contraction, relaxation events take place: Ca^{2+} is transported outside the cell via NCX and Ca^{2+}-ATPase and is transported back into the SR via SERCA(Ca^{2+}-ATPase of the SR). In cardiac and skeletal muscle, sorcin upon calcium binding interacts via its C-terminal domain with:

α1 subunit of the L-type voltage-dependent Ca^{2+} channel → slower inactivation;
Ryanodine receptor (RyR) → inactivation;
Na^{+}-Ca^{2+} exchanger (NCX) → activation;
SERCA→ activation.

Sorcin therefore lowers Ca^{2+} concentration in the cytosol, regulating important cardiac channels, thereby inhibiting excitation and activating relaxation processes.

Sorcin is emerging considered an important marker for cancers and for Multidrug resistance (MDR), and may participate in the MDR phenotype. Sorcin overexpression increases the MDR phenotype. Inhibition of sorcin expression leads to reversal of drug resistance. In particular, sorcin interacts with kinases such as PKA, CaMKII, and with CKII: phosphorylation participates in regulating sorcin and sorcin targets.

Sorcin is overexpressed in the brain, and is involved in the development of specific structures of the central and peripheral nervous systems. We are characterizing the interactions of sorcin with several targets in the neuronal cells, by using protein arrays, co-IP, Surface Plasmon Resonance. Sorcin interacts in a calcium-dependent fashion with many different targets, among which RyR2, RyR3, NCX, L-type voltage dependent Ca^{2+} channel, presenilin 2, α-synuclein, NMDA receptor, annexin 7 and 11, Rab11. Sorcin is therefore involved in calcium-dependent regulation of neuronal activity.

P4.9

Oscillatory Ca^{2+} dynamics and cell cycle resumption at fertilization in mammals: a modelling approach

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Fertilization in mammals is accompanied by repetitive Ca^{2+} increases in the egg cytoplasm, leading to exit from meiosis and entry into the first embryonic cell cycle. Sperm-egg fusion is associated with the delivery into the egg of PLCζ, a Ca^{2+}-sensitive phospholipase C that initiates Ca^{2+} oscillations by a well-understood mechanism (Saunders *et al.*, 2002; Berridge *et al.*, 2003). On the other hand, the network of cyclin-associated kinases driving the early embryonic cell cycle has also been well characterized (Kubiak *et al.*, 2008). However, the signal transduction pathway linking the Ca^{2+} changes to the activities of the cell-cycle related kinases is not fully elucidated. It is well established that the interaction between these 2 oscillatory phenomena involves the activation of calmodulin-dependent kinase II (CaMKII). Here, we present a computational model that we have developed (Dupont *et al.*, 2010) to investigate the mechanism by which cell cycle resumption can be sensitive to the temporal pattern of Ca^{2+} increases. Using a description of CaMKII activation that reproduces the frequency sensitivity of this kinase (De Koninck and Schulman, 1998; Dupont *et al.*, 2003), simulations confirm that Ca^{2+} spikes are accompanied by in phase variations in the level of CaMKII activity and suggest that in most mammalian species Ca^{2+} spikes are well suited to maximize CaMKII activation. The full model assumes that CaMKII brings about a decrease in the level of cyclinB-cdk1 by two pathways, only one of which is CSF-dependent. Parameters are selected to account for the experimental observations where mouse eggs were artificially activated by different Ca^{2+} stimulatory protocols. The model is then used in the context of 'assisted oocyte activation (AOA)' in humans to investigate why the best rates of successful activation are obtained when eggs are submitted to two applications of Ca^{2+} ionophore. This type of Ca^{2+} stimulation has been successfully used (Heytens *et al*, 2009) in protocols of intracytoplasmic sperm injection in the case of patients suffering from globozoospermia (i.e. who do not express PLCζ).

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P4.10

cAMP affects Ca^{2+} signaling in salivary glands of the blowfly, *Calliphora vicina*

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Fluid secretion in the salivary glands of the blowfly, *Calliphora vicina*, is induced by the neurohormone serotonin (5-HT). 5-HT simultaneously activates the $InsP_3/Ca^{2+}$- and the cAMP-signaling pathways (Berridge & Heslop, 1981), whereby threshold concentrations of 5-HT induce *intra*cellular Ca^{2+}-oscillations and *inter*cellular Ca^{2+}-waves (Zimmermann & Walz, 1997). We studied the influence of cAMP on the spatiotemporal pattern of the Ca^{2+}-oscillations and -waves, using isolated glands loaded with the Ca^{2+}-sensitive fluorescent dye Fura-2. We found:
(1) Cinanserin inhibits the 5-HT receptor that couples to the cAMP signaling pathway. Cinanserin decreases the frequency of intracellular Ca^{2+}-oscillations induced by threshold concentrations of 5-HT significantly.
(2) In the presence of cinanserin, the normal frequency of the 5-HT-induced Ca^{2+}-oscillations can be reconstituted by addition of the membrane-permeable cAMP analog 8-CPT-cAMP.
(3) Moreover, addition of 8-CPT-cAMP to sub-threshold concentrations of 5-HT elevates the Ca^{2+} spike frequency significantly.
(4) The protein kinase A inhibitors Rp-8-CPT-cAMPS and H-89 mimic the effects of cinanserin. Thus, the influence of cAMP on intracellular Ca^{2+}-oscillations is mediated by a PKA.
(5) The inhibition of 5-HT-induced cAMP formation by cinanserin, the addition of 8-CPT-cAMP in the presence of cinanserin, or PKA-inhibition by Rp-8-CPT-cAMPS affect the spatial pattern of the Ca^{2+}-signals as well, causing either spatially non-synchronized oscillations, distinct intercellular waves, and/or global beating.
This suggests that a delicate balance between the $InsP_3/Ca^{2+}$- and the cAMP-signaling pathways activated in parallel strongly affects the spatiotemporal pattern of intracellular Ca^{2+}-signaling. These observations supplement our previous findings that cAMP sensitizes, PKA mediated, $InsP_3$-induced Ca^{2+}-release from the ER (Schmidt & Baumann, 2008),and demonstrate distinct effects on intra- and inter-cellular Ca^{2+} signaling.

Acknowledgements:

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P4.11

Inhibitors of Bcl-2 protein family induce CICR and deplete Ca^{2+} stores in pancreatic acinar cells

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Small peptide inhibitors BH3I-2' and HA14-1 are known to disrupt interactions between Bcl-2 family proteins. We performed cytosolic and endoplasmic reticulum $[Ca^{+2}]$ measurements and found that the inhibitors of Bcl-2 protein interactions caused a slow and complete release of intracellular calcium stores. $[Ca^{+2}]$ release was attenuated by inhibitors of IP_3Rs and RyRs as well as clamping $[Ca^{2+}]$ with BAPTA-Ca2+ buffers. Inhibition of IP_3Rs and RyRs also dramatically reduced apoptosis induction. CICR induced by different doses of BH3I-2' in Bcl-2 overexpressing cells was markedly decreased compared to control. The results suggest that Bcl-2 proteins regulate calcium release from the intracellular stores through CICR dependent activation of IP_3Rs and RyRs.

P4.12

A coupling of mitochondria to store-operated Ca^{2+}-signaling sustains constitutive activation of protein kinase B/Akt and augments survival of malignant melanoma cells

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Mitochondria are emerging as a major hub for cellular Ca^{2+}-signaling [1], though their contribution to Ca^{2+}-driven growth- and survival-promoting events in cancer is not completely understood [2]. Here employing flow cytometry to monitor mitochondrial and cytosolic Ca^{2+}, we assessed trans-mitochondrial Ca^{2+}-transport triggered by store-operated Ca^{2+}-influx (SOC) and SOC function in malignant *vs.* non-malignant B16BL6 melanoma clones. Remarkably, both Ca^{2+}-fluxes were accelerated in the malignant B16BL6 cells expressing high levels of constitutively active protein kinase B/Akt (PKB). Interruption of trans-mitochondrial Ca^{2+}-transport in these cells by an antagonist of the Na^+/Ca^{2+}exchanger, CGP-37157, virtually abolished SOC, inactivated PKB, retarded cell growth and increased vulnerability to apoptosis. Direct blockade of the SOC by silencing Stim1 led to a similar inhibition of PKB, indicating that the crosstalk between SOC and mitochondria is essential to preserve PKB in constitutively active state. Finally, the retraction of mitochondria from sub-plasmalemmal microdomains triggered by transient over-expression of Fis1 inhibited SOC-mediated trans-mitochondrial Ca^{2+}-transport, Ca^{2+}-influx via SOC and PKB activity. Neither CGP-37157 nor Fis1 induced cell death or mitochondrial damage in the time window, within which Ca^{2+}- and PKB-measurements were performed. Taken together, our data show that tight functional and spatial coupling of mitochondria to the SOC is essential for the robust cellular Ca^{2+}-responses and down-stream signaling critical for apoptosis-resistance and accelerated growth of melanoma cells.

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P4.13

Caldendrin is intracellularly translocated by interaction with recoverin

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Caldendrin and recoverin are Ca^{2+}-sensor proteins operating in neuronal systems. Recoverin is known to regulate its target rhodopsin kinase in photoreceptor cells of the vertebrate retina in a Ca^{2+}-dependent manner. A light induced translocation of recoverin from the photoreceptor cell outer segments to the synapses was observed, while rhodopsin kinase did not translocate, indicating the existence of binding partners of recoverin different from rhodopsin kinase. In search of novel interaction partners of recoverin, we identified the neuronal Ca^{2+}-sensor protein caldendrin by employing a recoverin-affinity column. Caldendrin and recoverin are co-localized in a subset of bipolar cells in the retina and in the pineal gland as revealed by immunofluorescence studies. Pull-down-assays and surface plasmon resonance studies indicated that the recoverin-caldendrin-complex was formed in the presence of Ca^{2+} with low to moderate affinity. Importantly, caldendrin *in vitro* built a Ca^{2+}-independent homodimer. To study the cellular distribution, COS-7 cells were co-transfected with fluorescently labeled recoverin and caldendrin. Time lapse fluorescence microscopy revealed that the increase of intracellular Ca^{2+} facilitated the translocation of fluorescently labeled caldendrin to intracellular membranes. This process was apparently attributed to the complex formation with recoverin.

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P4.14

Regulation of autophagy by calcium

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Calcium is a universal second messenger regulating many physiological functions in the cell, such as secretion, contraction, metabolism, gene transcription and cell death, and implicated also in pathological processes. Calcium has been also described as an important regulator of autophagy.
Autophagy is an essential process of cellular self-digestion that allows cell survival under certain stress conditions and represents an evolutionary highly conserved and critical starvation response pathway. Changes in cytosolic calcium levels have been implicated to regulate the induction of autophagy. However, conflicting results have been achieved in studies that focused on the role of cytosolic calcium in the regulation of autophagy. Recent studies demonstrated that cytosolic calcium triggers signaling pathways that activate autophagy. In contrast, other studies provided evidence that the increase in cytosolic calcium should inhibit autophagy.
Here we show that intracellular calcium homeostasis is affected by starvation stress, and describe the subsequent signalling pathways activated by this second messenger, leading to changes in autophagy activity. In this regard, we give an account of the close relationship between calcium and starvation stress in the regulation of autophagy.
The present work provides new insights on the role of calcium in governing this process and show the importance of calcium homeostasis in autophagy response to stress conditions.

P4.15

Wnt signaling in the absence of calreticulin, a calcium buffering chaperone

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Calreticulin is a calcium binding chaperone involved in the regulation of intracellular calcium homeostasis, protein folding, gene transcription and cell adhesion. In mice, calreticulin deficiency is embryonic lethal due to disrupted cardiogenesis. Calreticulin-deficient embryonic stem (ES) cells are unable to differentiate into contracting cardiomyocytes in culture due to a disruption in the myofibril architecture as well as inhibition of the embryonic cardiac gene program. As Wnt signaling plays an important role during cardiac development, we investigated if Wnt signaling was affected in the absence of calreticulin. Unexpectedly, Wnt/β-catenin signaling was disrupted in calreticulin-deficient ES cells, but partially recoverable by treating with a GSK3β inhibitor. β-Catenin was mislocalized in calreticulin-deficient ES cells, bound to the plasma membrane. GSK3β phosphorylates β-catenin on serine/threonine residues, promoting degradation of the protein. GSK3β was hyperactive in the absence of calreticulin. Expression and activation of AKT, a kinase that inhibits GSK3β was decreased in calreticulin-deficient ES cells, as well as calcium-binding proteins; calmodulin, calcineurin, CaMKII and ERK1/2. We concluded that Wnt/β-catenin signaling was disrupted in the absence of calreticulin and may, in part, be responsible for embryonic lethality of the calreticulin-deficient mouse. To elucidate the role these genes play in Wnt/β-catenin signaling, we utilized a RNA interference library screen to examine the kinases and phosphatases and how they regulate Wnt/β-catenin signaling. The primary screen identified three categories of hits: genes that when silenced, inhibit Wnt/β-catenin signaling, genes that when silenced, activate Wnt/β-catenin signaling and genes that have no effect on downstream Wnt/β-catenin signaling. Analysis of the hits yielded 69 high confidence (HC) genes that when silenced, either inhibited or activated Wnt/β-catenin signaling; 54 of these HC genes had not been previously associated with Wnt signaling. Partitioning the genes into functional classes revealed multiple pathways, including amino acid metabolism, carbohydrate and lipid metabolism, post translational modification, cellular growth and proliferation and cell death. By coupling Wnt/β-catenin reporter activity with siRNA screening, we have identified several novel regulators of the Wnt/β-catenin signaling pathway in mouse ES cells, potentially linking a deficiency in calreticulin to abrogation of Wnt/β-catenin signaling.

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P4.16

Initiation of phagocytosis in protist parasite *Entamoeba histolytica* requires participation of EhC2PK and EhCaBP1

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Protist parasite *Entamoeba histolytica* is an etiological agent of amebiasis, a major cause of morbidity and mortality in developing world. *E. histolytica* trophozoites ingest microorganisms in the large intestine, as well as red blood cells and apoptotic immune cells during tissue invasion. Phagocytosis plays an essential role in growth and has been shown to be one of the key virulence determinants in amebiasis using both *in vitro* and *in vivo* models. Our laboratory is interested in deciphering the mechanisms of initiation of phagocytosis in *E. histolytica*. We are using phagocytosis of red blood cells or erythrophagocytosis as a model. EhCaBP1, an EF hand containing calcium binding protein, accumulates at phagocytic cups during erythrophagocytosis. Down regulation of EhCaBP1 expression inhibits erythrophagocytosis. Since EhCaBP1 was found to colocalize with actin at the phagocytic cups, direct binding of EhCaBP1 to F and G-actin was checked. It was found to bind specifically to both G- and F-actin in a Ca^{2+} dependent manner. However, the recruitment of EhCaBP1 to the phagocytic cups does not require its ability to bind Ca^{2+} as a Ca^{2+}-binding dead mutant still accumulated at the cups. Therefore, it appears that EhCaBP1 is recruited not through actin. Our search for a protein that may be recruiting EhCaBP1 to the cups led to identification of a C2 domain containing kinase (EhC2PK) as possible candidate. EhC2PK is a EhCaBP1 binding protein and this binding is Ca^{2+} independent. However, Ca^{2+} is required for EhC2PK to bind to lipid bilayer. The protein is localised to the phagocytic cups and down regulation of expression leads to inhibition of phagocytosis. Both EhCaBP1 and EhC2PK are absent in matured phagosomes. Our results suggest that during initiation of phagocytosis EhC2PK is first recruited to the site and this process is Ca^{2+} dependent. Subsequently EhCaBP1 is recruited in a Ca^{2+} independent manner. Once phagosomes are formed both EhCaBP1 and EhC2PK leave and only actin remains. Since both EhCaBP1 and EhC2PK are found only in *E. histolytica* so far, it appears that this parasite displays a novel mechanism of initiation of phagocytosis.

P4.17

Calcium affects cAMP signaling in salivary glands of *Calliphora vicina*

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Fluid secretion in salivary glands of the blowfly, *Calliphora vicina*, is induced by the neurohormone serotonin (5-HT). Binding of 5-HT to at least two distinct G-protein-coupled receptors leads to co-activation of the IP_3/Ca^{2+} and the cAMP signaling pathways (Berridge & Heslop, 1981). Interestingly, there is mounting evidence for extensive cross-talk mechanisms between both pathways.

Previous studies revealed a Ca^{2+}-dependent increase in intracellular cAMP that somehow involves calcineurin (Voss *et al.* 2010). Possible targets of this interaction are enzymes that govern the intracellular cAMP concentration – adenylyl cyclases (AC) and phosphodiesterases (PDE). Provided that the Ca^{2+} dependent rise in cytosolic cAMP is mediated by inhibition of PDE, one should be able to mimic this effect by pharmacological inhibition of PDE. However, this is not the case. In addition, the activity of phosphodiesterases in gland homogenates seems to be largely independent of Ca^{2+}. On the other hand, we were able to demonstrate a Ca^{2+}/calmodulin-dependent activation of adenylyl cyclases in membrane preparations. Calcineurin seems not to be involved in the regulation of both enzymes, at least *in vitro*.

In summary, we were able to identify an AC as a point of interaction between both signaling pathways. Nevertheless, the intracellular target of calcineurin still remains elusive. Subsequent experiments aim to identify the AC on the molecular and biochemical level.

Acknowledgements:

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P4.18

Role of STIM1 and store-operated calcium entry in cell death

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Alteration of the endoplasmic reticulum (ER) Ca^{2+} content has been linked to cell death in diverse paradigms. Stromal interaction molecule 1 (STIM1) is a major regulator of the ER Ca^{2+} content, which rearranges close to the plasma membrane in response to store depletion where it mediates store-operated Ca^{2+} entry (SOCE) from the extracellular space. A role for STIM1 in cell death has not been described despite its initial discovery as a growth-suppressing factor involved in rhabdomyosarcoma development. In this contribution, we investigated the effect of STIM1 on cell death.

ER stress was elicited by inhibition of SERCA ATPases using thapsigargin or inhibition of N-glycosylation by tunicamycin and oxidative stress by depletion of the intracellular antioxidant glutathione. We used mouse embryonic fibroblasts deficient in STIM1 (KO) and wildtype (WT) cells, KO cells stably transfected with vector, STIM1 or dominant-active STIM1, and HEK293 cells transfected with STIM1 and control siRNA. In addition, we studied the effect of pharmacological inhibition of SOCE on cell survival in WT and KO cells using 2-APB, SKF and ML9. Unexpectedly, we found a quite dramatic difference between ER stress and oxidative stress caused by glutathione depletion.

STIM1 deficiency increased the susceptibility to glutathione depletion but protected against ER stress. Pharmacologic inhibition of SOCE markedly reduced cell death induced by glutathione depletion but had no effect on ER stress. We concluded that STIM1 and SOCE are involved in susceptibility against glutathione depletion. To investigate this in further detail we analyzed the ionomycin and thapsigargin-releasable Ca^{2+} pool using single cell Fura2 Ca^{2+} imaging and the mitochondrial Ca^{2+} content using Rhod2AM staining in KO and WT cells. STIM1 KO cells exhibited a slightly increased cytosolic Ca^{2+} peak in response to thapsigargin or ionomycin, whereas mitochondrial Ca^{2+} was clearly increased in STIM1 KO cells, which was accompanied by a reduced mitochondrial membrane potential.

Our results suggest a novel role for STIM1 and SOCE in susceptibility to oxidative stress elicited by glutathione depletion, which is most probably related to the effects on mitochondrial Ca^{2+} homeostasis and functioning. It remains unclear why STIM1 deficiency leads to an increased mitochondrial Ca^{2+} level, but this could be related to alterations of mitochondrial and ER morphology and tethering.

P4.19

Molecular characterisation of endo-lysosomal two-pore channels

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Nicotinic acid adenine dinucleotide phosphate (NAADP) is the most potent intracellular Ca^{2+}-mobilising messenger so far discovered. Recently the two-pore channel (TPC) family of ion channels have been identified as molecular targets for NAADP [1, 2]. TPCs have been localized to the endo-lysosomal system where their activation by NAADP results in a "trigger" release of calcium which is subsequently amplified by calcium-induced calcium release from endoplasmic reticulum calcium stores. Little is known however about the molecular nature of these proteins in animal cells. We present the molecular characterisation of human TPCs and TPCs from the sea urchin [3], an extensively used model organism for study of NAADP mediated signalling. Our data provide new molecular insight in to the actions of NAADP.

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P4.20

Ca^{2+} mobilization in T-lymphocytes by 8-Br-*N*1-cIDPR, an analogue of the second messenger cADPR

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cADPR (cyclic ADP-ribose) is a universal Ca^{2+} mobilizing second messenger [1]. In T cells cADPR is involved in sustained Ca^{2+} release and also in Ca^{2+} entry [2, 3]. Potential mechanisms for Ca^{2+} entry include either capacitative Ca^{2+} entry, secondary to store depletion by cADPR or direct activation of the non-selective cation channel TRPM2 (transient receptor potential cation channel, subfamily M, member 2) [2, 4]. Here we characterize the molecular target of the membrane-permeant cADPR agonist 8-Br-*N*1-cIDPR (8-bromo-cyclic IDP-ribose) [5-7].

8-Br-*N*1-cIDPR induced Ca^{2+} signalling in both the humane T-lymphoma cell line Jurkat and in primary rat T cells. In Jurkat T cells we demonstrated that the Ca^{2+} signal consisted of Ca^{2+} release and Ca^{2+} entry. As expected Ca^{2+} release was sensitive to the ryanodine receptor blocker Ruthenium Red whereas Ca^{2+} entry was inhibited by the Ca^{2+} entry blockers Gd^{3+} and SKF-96365. In presence of the cADPR antagonist 8-Br-cADPR (8-bromo-cyclic adenosine diphosphoribose) Ca^{2+} release was partially and Ca^{2+} entry fully inhibited. Two mechanisms may be involved in inhibition of Ca^{2+} entry, either the diminished Ca^{2+} release resulted in inhibition of capacitative Ca^{2+} entry or direct activation of Ca^{2+} entry by TRPM2 was inhibited by 8-Br-cADPR.

To analyse a potential role for TRPM2 in Ca^{2+} entry mediated by 8-Br-*N*1-cIDPR, TRPM2 overexpressing HEK (human embryonic kidney)-293 cells were stimulated with 8-Br-*N*1-cIDPR; however, no significant differences to control cells were observed. Similarly, direct analysis of TRPM2 currents by patch-clamp analysis did not reveal activation of TRPM2 by 8-Br-*N*1-cIDPR. Kolisek et al. [4] showed that cADPR acts together with ADPR as co-activator of TRPM2. A small effect attributable to cADPR was observed when it was co-infused with a low concentration of ADPR. In contrast, replacement of cADPR by 8-Br-*N*1-cIDPR in such experiments did not evoke TRPM2 currents.

Taken together, 8-Br-*N*1-cIDPR appears to be the first cADPR agonist affecting Ca^{2+} release and secondary Ca^{2+} entry, but without effect on TRPM2.

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P4.21

A C-terminal domain of Bax inhibitor-1 displays Ca^{2+}-channel pore properties

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Bax inhibitor-1 (BI-1) is a highly conserved transmembrane protein identified as a suppressor of Bax-induced cell death in yeast (Xu & Reed, 1998). It localizes mostly into the ER membrane with its N- and C-terminal ends facing the cytoplasm. BI-1 is known to interact with the anti-apoptotic proteins Bcl-2 and Bcl-X_L but not with Bax itself. BI-1 inhibits the accumulation of ROS and regulates the UPR by binding to IRE1α (Lisbona *et al.* 2009). In addition, there is evidence that BI-1-overexpressing cells have lower $[Ca^{2+}]_{ER}$. The exact mechanism by which BI-1 regulates intracellular Ca^{2+} homeostasis is still unresolved, but it seems to involve the C-terminus of BI-1 (Westphalen *et al.* 2005).

We developed a set of peptides corresponding to a C-terminal domain of BI-1, CTP2 (aa 198-217), and analyzed their effects in unidirectional $^{45}Ca^{2+}$ fluxes in permeabilized mouse embryonic fibroblasts (MEFs). This approach revealed that CTP2 is able to provoke Ca^{2+} release from the inositol 1,4,5-trisphosphate (IP_3)-sensitive ER-Ca^{2+} stores with an EC_{50} of about 30 μM and an EC_{100} of 80 μM. Similar responses were observed in BI-$1^{-/-}$ MEFs and in DT40 cells lacking all three IP_3 receptor (IP_3R) isoforms, indicating that the CTP2-induced Ca^{2+} release is independent of both BI-1 and IP_3Rs. Secondary-structure predictions (I-TASSER) suggested that CTP2 forms an α-helical structure, which can be disrupted by targeted Gly mutations. With these Gly mutants, we were able to abolish the CTP2-induced Ca^{2+} release. Also, using artificial lipid bilayers, we showed that the CTP2 peptide is able to form Ca^{2+} pores independently of any other Ca^{2+}-release mechanism. Specific Ala mutants of CTP2 peptide did not provoke Ca^{2+} release, leading to the identification of critical residues involved in the channel-properties of the peptide. Finally, we investigated the properties of CTP2 peptides derived from the BI-1 of lower organisms. The analysis revealed that CTP2 peptides derived from insects to mammals were able to promote Ca^{2+} release, whereas peptides from plant and yeast orthologs were not. This suggests that BI-1 has a specific function in Ca^{2+} signaling that developed during evolution.

To conclude, our results indicate that the C-terminal part of BI-1 has a potency of Ca^{2+}-channel pore formation provoking a Ca^{2+} leak from the ER.

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P4.22

C-terminal segment of recoverin as a built-in modulator of neuronal calcium sensor functioning

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Neuronal calcium sensors (NCSs) are the family of EF-hand Ca^{2+}-binding proteins capable of recognition and regulation of effector enzymes in a Ca^{2+}-dependent manner, thus taking part in a number of signaling events in neurons. In spite of a high degree of structural similarity, NCSs demonstrate high selective recognition abilities for their specific target proteins and display different Ca^{2+}-sensitivities. Based on the sequence alignment, we assumed that a variable sequence in C-terminus of NCS proteins, the so-called "C-terminal segment", is a structural element which confers the specific Ca^{2+}-dependent functional properties on NCSs. To prove this assumption a photoreceptor Ca^{2+}-binding protein recoverin was taken as a model of NCS to study the role of the C-terminal segment in the NCS functionality. A number of recoverin mutants lacking the C-terminal 6, 10, 12, 14, 15, 16 or 18 amino acids, or containing P190G, Q191A, K192A, and V193G substitutions were generated by site-directed mutagenesis. The loss of fourteen or less C-terminal amino acids in recoverin, as well as all the point substitutions introduced, do not cause any destabilization of the protein structure as revealed by temperature dependence of the tryptophan fluorescence of recoverin. The further truncation of C-terminus of recoverin leads to substantial decrease in thermal stability of the protein. Using mutants obtained, it was demonstrated that the E^{189}PQKVKEK^{196} sequence in the C-terminal segment of recoverin is involved in the mechanism of its Ca^{2+}-myristoyl switch underlying the ability of recoverin to bind to photoreceptor membranes. Moreover, using affinity chromatography (pull down assays) and surface plasmon resonance measurements, it was shown that the 13 C-terminal amino acids of recoverin are necessary for the interaction of recoverin with its intracellular target, rhodopsin kinase. The latter result suggests the presence of a novel rhodopsin kinase binding site in recoverin in addition to the well-known site in the recoverin hydrophobic pocket. Based on the data obtained, we propose a novel role of the C-terminal segment as a sequence critical for the specific Ca^{2+}-dependent recognition of the target enzymes by members of the NCS family.

P4.23

$P2Y_2$ receptor activates signaling pathways which compensate Rho-kinase inhibition in glioma C6 cells

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Signaling pathways related to Rho family proteins and calcium response control the functional state of actin cytoskeleton, mainly by regulating the activity of two actin binding proteins: cofilin and myosin II. We have found that the effect of Rho-kinase activity inhibition can be reversed by stimulation of $P2Y_2$ receptor in glioma C6 cells. In current study we attempt to answer the following question: what signaling pathways induced by $P2Y_2$ receptor activation are involved in compensation of changes caused by Rho-kinase inhibition?

We have investigated changes in the level of phosphorylated cofilin and myosin II and compared them with the cell shape and F-actin organization. We have observed that ROCK inhibition decreases cofilin and myosin II phosphorylation. The effect observed on the subcellular level after ROCK inhibition is clearly visible in cell morphology and migration. We show here that UTP stimulation of the $P2Y_2$ receptor increases the level of phosphorylated cofilin and MLCs in glioma C6 cells with inhibited Rho-kinase and that these changes are responsible for restoration of the proper state of examined cells.

We suggest that:

— G_o-mediated Rac activation and Rac/PAK/LIMK pathway may be responsible for cofilin phosphorylation since inhibition of Rac activity by NCS 23766 negates this process.

— A myosin light chain phosphorylation is increased by calcium dependent MLCK - activated probably when calcium response is evoked by PLC activation mediated by G_q.

— G_{12}-mediated Rho activation is important for stimulation mDia which is responsible for actin polimerization. Both processes: MLC phosphorylation and actin polimeryzation are crucial for stress fibers reconstruction.

Further investigations are necessary for verifying these assumptions.

P4.24

Elevated $[Ca^{2+}]_c$ and calcineurin A modulate expression pattern of PMCA and of proteins involved in catecholamine secretion in PC12 cells

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PC12 cell line that originated from *phaeochromocytoma* is characterized by an excessive Ca^{2+}-dependent secretion of catecholamines. PC12 cells are equipped with various types of Ca^{2+} transporting systems. One of the most important Ca^{2+} transport systems maintaining low $[Ca^{2+}]_c$ consists of plasma membrane Ca^{2+}-ATPases. The expression profile of PMCA is regulated by calcium ions. The presence of particular PMCA isoforms and their splicing variants may affect functioning of the secretory apparatus.

Our experimental model consisted of PC12 cells stably transfected with plasmids carrying oligonucleotides against PMCA2 or PMCA3. These cells were characterized by aberrant calcium homeostasis as manifested by permanently increased $[Ca^{2+}]_c$. The sustained increase in $[Ca^{2+}]_c$ changed the level of certain PMCA isoforms and their alternative splicing variants, as well as the level of NCX isoforms. Most probably, increased $[Ca^{2+}]_c$ altered signal transduction mediated by calcineurin (PP2B), which activate several transcription factors. Immunocytochemistry analysis and co-immunoprecipitation assay revealed co-localization of PMCA4 with PP2B, which may potentially affect PP2B activity. The PP2B level increased, especially in the plasma membrane fraction. Application of cyclosporine A and FK506 (PP2B inhibitors) showed that inhibition of PP2B activity affected PMCA4 and NCX1 expression.

Suppression of either PMCA2 or PMCA3 significantly decreased catecholamine secretion. It was correlated with disordered gene expression and altered cellular localization of some proteins, e.g. Rab3A, ARF6, AnxA6, AnxA4, AnxA2, VAMP2, rabphilin, DBH, and CgA. These proteins are involved in the regulation of catecholamine secretion in a Ca^{2+}-dependent manner. The obtained results suggest that PP2B regulates expression of genes encoding the majority of proteins involved in secretion, including its own gene. The observed changes were triggered by local $[Ca^{2+}]_c$ changes which depended on the level and cellular localization of PMCA isoforms.

In conclusion, calcium signaling in PC12 cells with either PMCA2 or PMCA3 suppression and disturbed catecholamine secretion underwent global reorganization. Very fine changes of $[Ca^{2+}]_c$ led to unusual changes within the profile of the secretory machinery, which were correlated with changes in Ca^{2+} transporters expression. Calcineurin plays a crucial role in the modulation of gene expression pattern of Ca^{2+} transporters and secretory proteins.

P4.25

Structure/function analysis of the cytoplasmic tail domain of calnexin

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Calnexin, calreticulin, and ERp57, three endoplasmic reticulum (ER) resident chaperones, are responsible for the quality control (QC) of protein folding. Calnexin and calreticulin are functionally and structurally similar Ca^{2+} binding lectins that bind monoglucosylated carbohydrate on nascent polypeptides whereas ERp57 is an oxidoreductase. Together, these molecules ensure only properly folded and assembled protein complexes exit the ER. Calnexin and calreticulin share a functionally and structurally similar N+P domain in the lumen of the ER, responsible for binding nascent polypeptides. However, calreticulin is a luminal protein whereas calnexin spans the ER membrane and has a cytoplasmic tail (C-tail). The structure of calnexin's N+P domain has been solved and this portion has been shown to bind calcium. However, the structure and function of the C-tail has not yet been investigated. Here we show that the structure of the calnexin C-tail is a random coil, but it forms homodimers in solution. Ruthenium Red staining indicates the C-tail can bind calcium. The presence of calcium alters the structure of the C-tail, as assessed by circular dichroism (CD) analysis. Calcium binding to the C-tail could alter protein-protein interactions by potentially masking binding sites or changing its structure. This would have an impact on the C-tail as a signaling molecule. Using yeast two hybrid analysis we identified UBC9, a sumoylation E2 ligase, as a calnexin C-tail interacting protein. We show that the C-tail of calnexin can be sumoylated *in vitro*. This highlights the calnexin C-tail as a modifiable signaling entity that could provide communication between the cytoplasm and the ER lumen. Investigation of the structure and binding properties of the C-tail will add to the emerging concept of the C-tail as a crucial element in protein folding control and ER lumen to cytoplasm communication and signaling. Elucidating the structure and binding properties of the calnexin C-tail will help to understand the regulation of calnexin as a protein chaperone and other functions it may carry out.

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P4.26

Evidence of intracellular *S*-nitrosylation of S100A1 protein and its influence on three-dimensional structure of human *apo*-S100A1 protein

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S100A1 is a member of EF-hand containing Ca^{2+}-binding S100 protein family. It is predominantly expressed in brain and heart tissue where it plays a crucial role as a modulator of Ca^{2+} homeostasis, energy metabolism, neurotransmitter release and contractile performance. S100A1 effects on brain and heart function have been attributed to its direct interaction with a variety of target proteins. The physiological and pathophysiological relevance of S100A1 make it an important molecular target for future therapeutic intervention. Despite much research, the molecular mechanisms, through which S100A1 regulates cellular processes, are not fully understood. Here we present the data that S100A1 protein is endogenously, post-translationally modified by cysteine *S*-nitrosylation in PC-12 cells, a well established model system for studying S100A1 function. Previously, we have demonstrated that *S*-nitrosylation of Cys85 results in notable changes in the three-dimensional structure of bovine S100A1 protein [1]. In this work we describe high-resolution 3D structure of human *apo*-S100A1 protein with *S*-nitrosylated C-terminal Cys85 obtained at physiological ionic strength obtained from NMR data. For establish unique influence of *S*-nitroso group, our study also involves comparative analysis of 3D structure of *apo*-S100A1 protein with Cys85 thiol group in reduced state acquired under identical conditions. The results allow us better understand the molecular mechanisms regulated by S100A1 protein.

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P4.27

The role of TRPC2 in the function of rat thyroid cells

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Transient receptor potential (TRP) channels are widely expressed and have functions in many physiologically important processes. Perturbations in their expression or mutations in the channels have implications for diseases. In the present study we have studied which canonical TRP (TRPC) channels are present in rat thyroid FRTL-5 cells. We found that only TRPC2 was expressed. To investigate what thyroid specific processes that TRPC2 is important for, we utilized shRNA to decrease its expression. TRPC2 is, in FRTL-5 cells, activated downstream of receptor stimulation, and knocking down TRPC2 did not interfere with IP_3-mediated depletion of intracellular calcium stores. Electrophysiological data supports the calcium imaging results. Interestingly, the expression of thyroid-stimulating hormone receptor (TSHR) was increased in the TRPC2 knockdown cells. However, no change in the expression of thyroglobulin, sodium/iodide symporter (NIS) or thyroperoxidase (TPO) was observed. The production of cAMP was negatively regulated by TRPC2, since more cAMP was produced in TRPC2 knockdown cells. TRPC2 is important for proliferation and the progression of the cell cycle, since knocking down TRPC2 prolonged the G1/S cell cycle phase. This was due to upregulation of the tumor suppressor p53 and cyclin-dependant kinase inhibitors p27 and p21. In addition, the phosphorylation of p42/44 MAPK was also reduced in TRPC2 knockdown cells. The maturation of thyroglobulin was also affected in cells were TRPC2 was knocked down. Probably due to improper folding and glycosylation, a part of thyroglobulin was retained in the ER. At least in part, this led to a decrease in the secretion of thyroglobulin from TRPC2 knockdown cells. The uptake of iodine was increased in TRPC2 knockdown cells, presumably as an effect of the increased expression of TSHR as there was no difference in NIS expression. In conclusion, TRPC2 is a major regulator of rat thyroid cell function.

P4.28

Vesicular trafficking of the Orai1 channel at rest and during cell division

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The egg's competency to activate at fertilization and transition to embryogenesis is dependent on its ability to generate a fertilization-specific Ca^{2+} transient. To endow the egg with this capacity, Ca^{2+} signals remodel during oocyte maturation including inactivation of the primary Ca^{2+} influx pathway store-operated Ca^{2+} entry (soce). Soce inactivation is coupled to internalization of the soce channel, Orai1. Here we show that Orai1 internalizes during meiosis through a caveolin- and dynamin-dependent endocytic pathway. Caveolin binds to Orai1 and we map a caveolin consensus binding site in the Orai1 N-terminus, which is required for Orai1 internalization. Furthermore, at rest Orai1 actively recycles between an endosomal compartment and the cell membrane, through a Rho-dependent endocytic pathway. A significant percentage of total Orai1 is intracellular at steady state. Store depletion completely shifts endosomal Orai1 to the cell membrane. These results define for the first time vesicular trafficking mechanisms controlling Orai1 subcellular localization at steady state, during meiosis and following store depletion.

P4.29

Calcium compartmentation and more specifically nuclear calcium controls the apoptotic-like cell death induced in tobacco cells by dihydrosphingosine (d18:0) in a ROS independent way

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We have recently shown that sphingoid long-chain bases (LCBs) are able to trigger nuclear calcium responses in tobacco BY-2 cells constitutively expressing the calcium probe aequorin in the nucleus (Xiong et *al.*, 2008, *Cell Calcium* **43:** 29–37). We further investigated the signalling pathway of these lipids on the tobacco cells by assessing the effect of the first member of the sphingolipid biosynthesis pathway, dihydrosphingosine or d18:0 (DHS) on BY-2 cells. We show here that this compound immediately induces a change in the sphingolipid metabolism followed by dose-dependent cytosolic and nuclear calcium variations. In addition, DHS induces a burst of Reactive Oxygen species (ROS). Upon 30 min of treatment, cell death symptoms are appearing and display apoptotic-like hallmarks such as cell shrinkage, chromatin condensation and caspase-like activities. Lanthanum chloride, a general blocker of calcium entry, suppresses the cellular calcium variations, the burst of ROS and the PCD induced by DHS. We thus evaluated the importance of ROS and of each calcium compartment in the signalling pathway leading to cell death. We report here that DL-2-amino-5-phosphopentanoic acid (AP5) and [(+)-dizocilpine] (MK801), two inhibitors of animal and plant ionotropic glutamate receptors, suppress DHS-induced cell death symptoms by selectively inhibiting the variations of nuclear calcium concentrations. We also show that suppression of ROS with the NADPH oxidase inhibitor diphenyleneiodonium chloride (*DPI*) does not impact on cell death. Altogether our work highlights the crucial role of nuclear calcium signature in controlling DHS-induced cell death in tobacco cells in a ROS-independent manner.

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P4.30

The novel calmodulin-binding site of the smooth muscle myosin light chain kinase

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Myosin light-chain kinase (MLCK) is composed from the N-terminal actin-binding domain, central catalytic domain, and C-terminal myosin-binding domain. MLCK has not only kinase activity to phosphorylate myosin regulatory light chain but also non-kinase activity (actin-binding etc.). We have already reported that the actin-binding activity regulated by Ca^{2+}-calmodulin (CaM). We generated the construct of kinase-dead that lost the CaM-binding site (M13) for kinase activation. The regulation by the Ca^{2+}-CaM for non-kinase activity remained though the mutant lost the CaM-binding of M13 site. We searched for the CaM-binding sites other than M13 domain by the technique of bioinformatics (CaM Target Database). As a result, we found a novel CaM-binding to N-terminal side of the M13 site. So, we expressed the peptide region of novel and M13 site by bacteria expression system and confirmed the CaM-binding activity by using the CaM-agarose and Surface Plasmon Resonance. In the *in vitro* experiment, the novel peptide binds with CaM. The novel CaM-binding domain was shown to be involved in the regulation of non-kinase activity by Ca^{2+}-CaM.

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P4.31

Calcium signaling triggered by NAADP in T cells determines cell shape and motility during immunological synapse formation

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Nicotinic acid adenine dinucleotide phosphate (NAADP), reviewed in [1], has been implicated as initial Ca^{2+} trigger in T cell Ca^{2+} signaling in T-lymphoma cells, but its role in formation and maintenance of the immunological synapse in $CD4^+$ effector T cells has not been analyzed. $CD4^+$ T cells are activated by the interaction with peptide-MHCII complexes on the surface of antigen (Ag)-presenting cells. Establishing an *in vitro* two-cell system including primary rat $CD4^+$ T cells specific for the CNS antigen myelin-basic protein and the rat astrocyte cell line F10 enabled us to mirror this activation process in vitro and to analyze Ca^{2+} signaling, cell shape changes and motility in T cells during formation and maintenance of the immunological synapse. After contact to Ag-MHCII complexes on the surface of astrocytes, T cells showed strong, Ag-dependent increases in global free cytosolic calcium concentration. In the absence of Ag, T cells exhibited only weak and delayed calcium signals. Further analysis of cell shape and motility revealed rounding and immobilization of T cells depending on the amplitude of the Ca^{2+} signal. The recently introduced NAADP-specific antagonist BZ194 [2, 3] effectively blocked Ca^{2+} signals in T cells evoked by the interaction with Ag-presenting astrocytes. BZ194 reduced the percentage of T cells showing considerable Ca^{2+} signals thereby supporting a proposed trigger function of NAADP for global Ca^{2+} signaling. Altogether, the data indicate a pivotal role for NAADP in early T cell signaling.

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P4.32

Changes in structure and dynamics of human *apo*-S100 protein upon calcium binding

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S100 is a multigenic family of calcium-modulated proteins of the EF-hand superfamily implicated in intracellular and extracellular regulatory activities. Most of S100 proteins exist in the form of antiparallely packed homodimers stabilized by nonbonding interactions. It is widely accepted that proteins from the S100 family are signalling proteins. S100A1 contains 93 residues per subunit and each subunit contains two calcium binding loops.

As was previously shown for rat S100A1 protein [1, 2], binding of calcium ions induces significant changes in the protein structure. The goal of our present study is to compare possible structural differences between human and rat proteins and to study how the protein backbone dynamics is affected by calcium binding.

Using a number of multinuclear 3D NMR techniques applied to the samples of ^{13}C,^{15}N double labeled *apo*- and *holo*-S100A1 human protein measured at 11.7 T, 16.4 T and 18.8 T we have assigned chemical shifts of almost all backbone and side chain N, C, and H nuclei (results for human *apo*-S100A1 were deposited in BMRB entry no. 16360).

For *apo*-S100A1 the structural information derived from chemical shifts was combined with distance constrains derived from ^{13}C and/or ^{15}N edited NOESY spectra and used to calculate its 3D structure with XPLOR and CYANA software. For *holo*-S100A1 the preliminary low resolution structure of the subunits was predicted on the basis of chemical shifts.

For both forms of human S100A1 protein ^{15}N nuclear magnetic relaxation data at three magnetic fields (9.4, 11,7 and 16.4 T) were obtained and their backbone dynamics was characterized. The *apo* form displays significantly increased mobility of both binding loops and of the linker joining the two calcium-binding EF-hand domains. In the *holo* form this mobility is partially restricted.

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P4.33

Investigation of Selenoprotein N1 interacting proteins

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Selenium is inserted into proteins as a selenocysteine (Sec) residue by recoding of UGA 'stop' codons by dedicated translational machinery (Hatfield & Gladyshev, 2002). Selenoprotein N1 (SEPN1), isoform 2, a calcium (Ca^{2+})-binding integral membrane selenoprotein of the endoplasmic/sarcoplasmic reticulum, bears one Sec residue. Robust expression of SEPN1 in fetal tissue muscle precursors decreases during development, suggesting a role in myogenesis (Petit *et al.*, 2003). SEPN1 may partake in myogenesis by oxidising ryanodine receptor (RyR) Ca^{2+} channel thiol groups, thereby altering Ca^{2+} homeostasis, since SEPN1 and RyR1 interact *in vivo* (Jurynec *et al.*, 2008). To gain further insights into SEPN1 function, this study employed two structural motifs of SEPN1 in peptide-protein pulldown assays. Sequence analysis of SEPN1 revealed the presence of an N-terminal Class II SH3 interaction motif. A biotinylated peptide (14-mer) corresponding to this motif, or a scrambled peptide negative control, were synthesised and used to probe solubilised microsomal fractions from A549 adenocarcinoma cells. One major interacting protein was reproducibly isolated and identified as complement component 1 Q subcomponent-binding protein (C1QBP) by MALDI-TOF spectroscopy. Secondly, biotinylated peptides (21-mers) incorporating the C-terminal putative catalytic motif of SEPN1 were synthesised, in which either of the redox-reactive residues (Sec or Cys) of this motif were substituted for a norvaline residue. Such peptides would be expected to 'trap' potential substrates of this motif. Rat skeletal muscle homogenates were probed with these peptides under normal physiological and oxidative stress conditions set by a defined glutathione redox couple ratio. Under normal physiological conditions, there was no major distinction between substrates trapped by either the Sec or Cys residues. Under conditions imitating oxidative stress, one major substrate was trapped by the Cys residue of the catalytic motif. MALDI-TOF analyses identified this substrate as creatine kinase M chain. Immunoprecipitations and immunofluorescence with antibodies specific for these candidate SEPN1 interaction partners reinforce evidence of *in vivo* association for the first time.

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P4.34

The ciliated protozoan, *Paramecium tetraurelia,* contains a plethora of Ca^{2+}-release channels — mainly novel types

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To understand in more depth Ca^{2+}-signaling in the ciliated protozoan, *Paramecium tetraurelia* [1], we have searched the database of the recently completed *Paramecium* genome [2]. This has revealed the presence of 34 genes encoding proteins related to mammalian Ca^{2+}-release channels of the ryanodine- or IP_3-receptor type (RyR, IP_3R). Phylogenetic analyses show that the 34 possible Ca^{2+}-release channels (CRCs) can be subdivided in six different groups (CRC-I to CRC-VI) [3], each showing characteristic features regarding their relationship to mammalian IP_3Rs and RyRs. Up to now two families of CRCs, CRC-II-1 (also designated as IP_3R_N) and CRC-IV-1 were analysed in more detail on a molecular level. CRC-II-1 channels possess all features of mammalian IP_3Rs when identified according to all molecular and functional criteria [4] – the only one esstablished in the protozoan kingdom. Antibodies against CRC-II-1 channels unexpectedly stain membranes of the contractile vacuole system. The CRC-IV-1 types differ, despite their highly conserved channel domains, in terms of the regulatory and the ligand binding domain, e.g. absence of an IP_3-binding domain and of any IP3 or ryanodine effects in vivo, yet sensitivity to the RyR agonist, 4-Cl-m-cresol. CRC-IV-1 types could be localized particularly to the outer side of membranes of cortical Ca^{2+} stores, the alveolar sacs, and additionally to membranes of the endoplasmic reticulum [3]. Functional analyses by gene silencing experiments reveal that knock down of CRC-IV-1 channels results in a reduced exocytotic capacity of dense core vesicles ("trichocysts") due to reduced Ca^{2+} signal generation upon stimulation, as found by Ca^{2+} imaging [3]. In contrast, silencing of CRC-II-1 leads to decreasing amounts of trichocyst contents proteins, suggesting that the biogenesis of trichocysts is affected due to altered Ca^{2+} homeostasis [4]. Meanwhile we have prepared antibodies against members of the other Ca^{2+}-release channel families which currently allows us their localization and further characterization.

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P4.35

Regulation of store-operated Ca^{2+} influx by the endoplasmic reticulum luminal environment

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Intracellular Ca^{2+} signaling typically entails release of Ca^{2+} from the endoplasmic reticulum (ER) lumen to the cytoplasm where it can regulate a variety of processes as diverse as fertilization, gene transcription, and apoptosis. This processes empties intracellular Ca^{2+} stores, necessitating their replenishment via a process known as store-operated Ca^{2+} entry (SOCE), whereby a single-pass ER membrane Ca^{2+} sensing protein, STIM1, signals a plasma membrane Ca^{2+} channel, Orai1, to allow for Ca^{2+} entry from the extracellular medium. The mechanism allowing STIM1 to respond to Ca^{2+} levels and later communicate with Orai1 relies on a Ca^{2+}-binding EF-hand and oligomerization domains, both luminal and cytoplasmic. Specifically, Ca^{2+} dissociation from the luminal EF-hand of STIM1 induces a conformational shift that favors homomerization, thought to be mediated by sterile alpha motif (SAM) domains in the ER lumen. Oligomerization of STIM1 triggers its movement to subplasmalemmal punctae, where it directly interacts with Orai1 to initiate SOCE. Though ER luminal oligomerization is known to be the triggering step of SOCE, the specific molecular dynamics have yet to be completely understood. We therefore decided to further investigate the luminal regulation of STIM1, hypothesizing that it was likely to be regulated by some ER luminal binding protein. Using a variety of biochemical and cell biological techniques, we identified luminal partners of STIM1 thereby modulating SOCE. Interactions between STIM1 and ER luminal components were demonstrated *in vitro* via surface plasmon resonance and *in vivo* via Forster resonance energy transfer (FRET). Use of ER chaperone-deficient cell models and single-cell Ca^{2+} imaging confirmed the importance of luminal regulation of STIM1 and its oligomerization. Further work is required to completely understand how STIM1 self-associates, specifically focusing on how changes in its structure affect its Ca^{2+}-dependent activation of STIM1 and regulation by ER luminal proteins.

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P4.36

S100A6 is required for nuclear translocation of Sgt1 — a heat shock regulated protein

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Sgt1 was originally identified in yeast cells where it was shown to be involved, *via* interaction with Skp1, in the CBF3 kinetochore complex and in the SCF ubiquitin ligase [1]. Later it was shown that Sgt1 binds some other ligands such as S100A6 [2] and Hsp90 [3]. The C-terminal fragment of Sgt1 (SGS) is responsible for binding of S100A6 while the CS and SGS domains of Sgt1 interacts with Hsp90. Interestingly, depending on $[Ca^{2+}]_i$ S100A6 attenuates the interaction between Sgt1 and Hsp90 [4]. Recently, it was shown that the Sgt1 protein has co-chaperone properties and is up-regulated by heat shock [5]. Since it is known that under stress conditions some proteins, including chaperones, translocate from the cytoplasm to the nucleus [6, 7], in this work we examined the changes in subcellular localization of Sgt1 due to heat shock with special focus on its nuclear translocation. We found that in heat-shocked cells the amount of Sgt1 increased in the nucleus and that this translocation was specific. Furthermore, considering that heat shock may induce changes in $[Ca^{2+}]_i$, we checked whether the calcium binding protein — S100A6 might have an effect on Sgt1 translocation depending on $[Ca^{2+}]_i$. We have found that in HEp-2 cells with diminished level of S100A6, due to stable transfection with siRNA against S100A6, nuclear translocation of Sgt1 occurred at a much smaller scale in comparison with cells expressing a normal level of S100A6. Moreover, translocation of Sgt1 was observed in HEp-2 cells treated with thapsigargin instead of heat shock. In contrast thapsigargin was ineffective in cells with diminished level of S100A6. To check whether the effect of S100A6 on Sgt1 nuclear translocation is exerted via regulation of Sgt1 phosphorylation we prepared S249A or S299A or double S249A/S299A mutants of Sgt1. The nuclear translocation of these mutants after heat shock in HEp-2 cells with normal and diminished level of S100A6 is now under investigation.

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P4.37

Calcium signaling influences early neural development

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Embryonic Stem (ES) cells are defined by their capacity of self-renewal and their potential to differentiate into any cell type. Calcium (Ca^{2+}) is an important intracellular second messenger that regulates many different cell functions, like proliferation and differentiation. Here we have examined the impact of Ca^{2+} signaling on differentiation of ES cells into neurons. Applying a protocol of neural differentiation from mouse ES cells, we followed the differentiation for 10 days with Ca^{2+} imaging, qPCR and immunocytochemistry. The spontaneous Ca^{2+} activity increased from day 6 of differentiation, when beta-III tubulin positive (TUJ1+) neurons emerged. Response to high KCl augmented from day 6 of differentiation, indicating a correlation between spontaneous Ca^{2+} activity and voltage dependent Ca^{2+} channels (VDCCs). Studies on the expression profiles of VDCCs during the course of differentiation using qPCR revealed an increase in expression of L-type VDCCs from day 6, whereas T-type VDCCs levels increased from day 4. Culturing cells in low or high K^+ concentration led to a difference in Microtubule-Associated Protein 2 (MAP2) and TUJ1 expression from day 6, suggesting a critical role for VDCCs in development. To further investigate the role of Ca^{2+} signaling on neural differentiation, Suramin, an ATP receptors inhibitor, and BAPTA treatments were applied from day 6 of differentiation. Cells treated with BAPTA showed increase in neuronal like cells (TuJ1+ and MAP2 +) and decrease in endodermal progenitors cells (GATA4+). Suramin treatment decreased MAP2 + cells and increased GATA4+ cells.Taken together, these data demonstrate that Ca^{2+} plays an important role in neural differentiation.

P4.38

Regulation of the expression and ligand-dependent activation of the epidermal growth factor receptor (EGFR) by calmodulin (CaM) in conditional CaM-knockout cells

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The epidermal growth factor receptor (EGFR) is a tyrosine kinase that controls cell proliferation, differentiation, cell survival and cell migration. This receptor is implicated in the pathogenesis of some solid tumors due to its overexpression and/or mutations resulting in enhanced ligand-independent signaling. We have demonstrated that the EGFR is a Ca^{2+}/calmodulin (CaM)-binding protein (see for review [1]), with a CaM-binding domain located at its cytosolic juxtamembrane region [2]. We have shown using CaM antagonists that the ligand-dependent activation of the EGFR could be facilitated by the Ca^{2+}/CaM complex in living cells [3, 4]. Recently, we have engineered chicken DT40 lymphoma B cell lines in which the two expressed CaM genes were deleted and one CaM I allele replaced by an inducible rat CaM III expression construct (Tet-off system) [5, 6]. Wild type and conditional CaM knockout DT40 cells were stably transfected with the human EGFR, and several clones expressing functional receptors were isolated. The addition of EGF to these cells induced autophosphorylation (activation) of the receptor and enhanced their proliferation rate, decreasing the doubling time from 10.8 ± 0.6 to 8.9 ± 0.2 h during exponential cell growth. Progressive downregulation of CaM upon tetracycline treatment first increased (≈ 2-fold) and thereafter decreased (to near basal level) total EGFR expression. The presence of KN93, an inhibitor of CaM-dependent protein kinase II (CaMKII), further enhanced (up to ≈ 5-fold) the total EGFR level when CaM was moderately low, suggesting that CaMKII downregulates EGFR expression. The EGFR at the plasma membrane also mirrored those changes in the presence and absence of KN93, albeit less intensely. In order to further understand the role of CaM in the ligand-dependent activation of the EGFR [3, 4], we will present results on how cellular depletion of CaM affects the tyrosine phosphorylation (activation) of the receptor. We conclude that the Ca^{2+}/CaM complex may be a pleiotropic regulator of the EGFR in living cells, as it affects the functionality of this receptor at multiple levels.

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P4.39

Analysis of NAADP metabolism

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Ca^{2+} release from intracellular stores is mediated by various second messengers including inositol 1,4,5-trisphosphate (IP_3), cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP). The most recently identified messenger among these is NAADP. It is also the most potent second messenger known to date and effectively mobilizes Ca^{2+} from intracellular stores at low nanomolar concentrations [1]. The NAADP signaling system has been demonstrated in a variety of mammalian tissues [2-4]. Moreover, NAADP has been claimed to provide a trigger Ca^{2+} signal which is then amplified by cADPR and IP_3 mediated signaling mechanisms [5].
Therefore precise regulation of the intracellular NAADP level is essential for functional Ca^{2+} signaling. However, the metabolic pathways responsible for the synthesis and degradation of NAADP in vivo remain to be elucidated.
The aim of our study was to characterize NAADP metabolism in HeLa cells. A NAADP degrading activity was detected in membrane preparations of HeLa cells. HPLC analysis revealed the dephosphorylation of NAADP to nicotinic acid adenine dinucleotide (NAAD). Pharmacological studies and determination of pH optimum indicated the newly identified enzyme activity might be an alkaline phosphatase. Expression of alkaline phosphatase in HeLa cells was verified by RT-PCR and western blot experiments. Contrary to NAADP the degradation product NAAD has no Ca^{2+} mobilizing effect [1]. The dephosphorylation of NAADP to NAAD would therefore provide an effective mechanism to terminate the NAADP mediated signal. Our studies provide new insights to elucidate the metabolic pathway of the Ca^{2+} mobilizing second messenger NAADP.

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P4.40

The Ca^{2+} relay model in vertebrate cone vision

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Among the five classes of neuronal Ca^{2+} sensor proteins (NCS proteins), two of them — Recoverin and the Guanylate cyclase-activating proteins (GCAPs) — are considered to be retina specific and play a crucial role in photoreceptor cells' adaptation to different background light intensities. In vertebrate rod photoreceptor cells two GCAP isoforms are expressed. Although these two isoforms adress to the same target molecules — Guanylate cyclases (GCs) – they possess different properties and Ca^{2+} sensitivities. The expression of two GCAP isoforms in the same cell has led us to propose a Ca^{2+} relay model, where the GCAP isoforms consecutively step into action, due to increasing light intensities and according to decreasing $[Ca^{2+}]_i$.

While rod photoreceptor cells operate at low light intensities, cone photoreceptor cells are responsible for daylight vision and exhibit more dynamic responses over a huge range of ambient illumination. Recent studies [1, 2] suggest that Ca^{2+} homeostasis in cone cells is more complex than in rod cells due to the different requirements of both cell types for adaptation. How do these results fit in our Ca^{2+} relay model? Are the differences in fluctuating Ca^{2+}-homeostasis in rods and cones mirrored in different sets of GCAPs with slightly different biochemical properties? To adress these questions the cone dominated retina of zebrafishes, where six GCAP isoforms (zGCAPs) are expressed, is an excellent model.

We heterologously expressed the six zGCAP isoforms and compared the biochemical properties of the purified proteins: All zGCAPs exhibit properties suitable for their role as Ca^{2+} sensors like Ca^{2+}-dependent differences in protease accessibility and Ca^{2+}-induced conformational changes monitored by tryptophan fluorescence measurements. Furthermore, all six isoforms were activators of membrane bound guanylate cyclases from bovine retina. The halfmaximal activation (IC_{50}-values) range from 30 nM (zGCAP1) to approx. 500 nM (zGCAP4) $[Ca^{2+}]_{free}$. Given the different expression pattern of zGCAPs in the retina, these results support a role for GCAPs with fine-tuned Ca^{2+}-sensing properties operating consecutively in rod and cone cells.

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P4.41

The role of Ca^{2+} binding and tyrosine phosphorylation in the essential survival functions of calmodulin (CaM) analyzed in a chicken DT40 cell line with artificially regulated CaM expression

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Calmodulin (CaM) is essential for survival of eukaryotic cells [1] yet no direct mechanism of its growth supporting activity in vertebrate cells has been described so far. CaM regulates the majority of its known binding-partners in a Ca^{2+}-dependent manner, although Ca^{2+}-independent interactions have been described. Tyrosine phosphorylation of mammalian CaM has been shown to affect target interaction and activation but its significance for cell proliferation is not yet known [2]. In vertebrates, CaM is expressed from several genes, which all code for the same protein, making it difficult to generate a model system where the effect of CaM gene deletion can be studied. Thus, the cellular survival function of CaM has only been established in genetic systems of lower eukaryotes [1, 3-5]. In order to investigate the essential function of CaM in vertebrates we have generated chicken lymphoma DT40 cell lines with artificially regulated CaM levels. DT40 cells are well suited for knock out experiments as homologous recombination occurs with relatively high frequency. These cells express CaM from two genes, CaMI and CaMII, the latter contributing the majority of CaM. In a first step, both alleles of the CaMII gene were knocked out. Decreasing CaM levels to 40% of wild type levels caused a lower growth rate as compared to wild type cells [6]. In a second step, one allele of CaMI was replaced by a rat CaMIII gene under the control of a tetracycline (tet) regulated promoter ($CaMII^{-/-}$, $CaMI^{-/ratCaMIII}$) and finally the second CaMI allele was deleted. This system is devoid of endogenous CaM and allows to downregulate rat CaM expression by addition of tet to the culture medium. We show that CaM is essential in vertebrate cells as addition of tet led to growth arrest and cell death after 6 days. Withdrawal of tet or stable ectopic expression of HA-tagged wild type CaM rescued the cells. In addition, experiments with cell lines stably expressing HA-tagged CaM mutants with impaired individual Ca^{2+}-binding sites or tyrosine phosphorylation sites (Tyr99 and Tyr138) are in progress and their effect on cell proliferation will be presented.

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P4.42

Regulation of TRPC2 calcium channels in thyroid cells

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Mammalian transient receptor potential cation channels (TRPCs), non selective Ca^{2+} channels, are putative receptor- and store-operated cation channels that play a fundamental role in the regulation of cellular Ca^{2+} homeostasis. Previous studies show that the phosphatase inhibitor calyculin A (caly A) evoked a marked increase in cytosolic free calcium concentration ($[Ca^{2+}]_i$). The increase in the cytosolic free calcium was blocked by 50 μM 2APB and 10 μM SKF96365. 2APB also blocked the cytosolic sodium entry indicating a presence of a non-selective channel permeable to both calcium and sodium. Electrophysiological data also supports these results. RT-PCR screening of the FRTL-5 cells revealed the presence of TRPC2 channels which is known to be highly localized to the dendritic tip of vomeronasal sensory neurons. The TRPC2 channels were knocked down using shRNA and further experiments indicated that TRPC2 mediated the caly A evoked calcium entry. Stimulating TRPC2 knockdown cells with ATP in calcium containing buffer evoked a decreased response in $[Ca^{2+}]_i$ compared with control cells. In a calcium-free buffer, there was no difference in ATP-evoked increase in $[Ca^{2+}]_i$ between control cells and TRPC2 knockdown cells. There was no difference in the thapsigargin (Tg) evoked calcium entry in knockdown cells compared to control cells. Hence here we show that in rat thyroid FRTL-5 cells, the TRPC2 channel seems to function only as a receptor-operated ion channel.

P4.43

Development of selective antagonists for NAADP in human platelets

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NAADP is a Ca^{2+}-releasing second messenger that acts to release Ca^{2+} from an acidic store, rather than the endoplasmic reticulum. Although NAADP is known to release Ca^{2+}, its role in Ca^{2+}-mediated activation and aggregation of human platelets is still relatively unknown. We investigated the role of NAADP signalling in platelet activation using existing antagonists, but found them to act with relatively low affinity compared to other systems. Using a computer-based screen, we identified a series of NAADP analogues that are related to NAADP in three-dimensional, but not two-dimensional, structure. We then went on to test these compounds for activity in human platelets. The screening procedure we used included a Ca^{2+} release assay and a [^{32}P] NAADP binding assay. Analogues were then tested in the process of agonist-induced platelet activation and aggregation. A number of compounds were found to selectively antagonise NAADP-induced Ca^{2+} release in human platelets. As the compounds are cell-permeant, they interfered with agonist-induced Ca^{2+} signals and platelet aggregation in whole cell assays. The identification of these novel antagonists of NAADP will have widespread utility within the field as studies extend to a wider range of cell types. Furthermore, we demonstrate that the NAADP receptor in platelets is a druggable target and we show that the NAADP receptor may represent a novel and important target for antithrombotic drugs.

P4.44

A minimal 3-dimensional model of an atrial myocyte with a realistic distribution of calcium release sites: a computational cell biology approach

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Here we develop a 3-dimensional model of an atrial myocyte with a cylindrical geometry. Transport of calcium within the cell is modelled by diffusion, with sarcoplasmic reticulum (SR) re-filling modelled with a linear pump. This is described mathematically with a linear second order parabolic partial differential equation (PDE). Calcium release is considered to act only at discrete points within z-planes via a calcium-induced-calcium release mechanism. We take this to be a threshold process that releases calcium from the SR at a constant rate for a fixed duration comparable to the lifetime of a calcium spark. These calcium release events are regarded as source terms in the linear PDE, triggered when cytosolic calcium exceeds threshold, which mimics calcium excitability. By solving the dynamics between release events (exploiting the linearity of the PDE) we formulate a minimal model solely in terms of behaviour at the release sites. This minimal description contains all the original biology and geometry of the full model, while being computationally inexpensive. The main overhead lies in the summation of release unit activity that contributes to the spatio-temporal evolution of the calcium concentration throughout the whole cell. In contrast to ventricular myocytes, atrial myocytes do not possess a T-tubule system and hence the issue of calcium wave propagation to the cell interior is much more important for subsequent cell contraction. Our computational model allows us to exhaustively probe the dependence of wave properties (speed, shape, path through the cell), on stimulus protocols, release strength, pump-rates, and values of the effective diffusion coefficient. Importantly we find that a diffusive gap, i.e. the absence of release sites, in the sub-sarcolemmal space (consistent with experimental findings) can lead to propagation failure of centripetal waves if the initial stimulus is too weak. Thus the model is useful for exploring the functional consequences (degree of myocyte contraction) of hormonal or electrical stimulation.

P4.45

S100A8 and S100A9 affect adhesion and migration of phagocytes *via* the microtubule based cytoskeleton

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The migratory properties of phagocytes allow their rapid accumulation at sites of injury and infection. The complex transmigration processes include rolling, adhesion and diapedesis are not fully understood, especially the ability of phagocytes to rapidly reorganize their cytoskeleton. Calcium-binding proteins are key elements in these signal transduction processes. We therefore investigated the role of the two phagocyte specific calcium-binding proteins S100A8 (MRP8) and S100A9 (MRP14) to clarify, whether these proteins are involved in the signaling cascades. Previously we could already show, that in activated monocytes complexes of S100A8/S100A9 co-localize with cytoskeletal components in a calcium and phosphorylation dependent manner. S100A8/S100A9 directly interacts with and stabilizes microtubules (MTs) in human monocytes. Furthermore, phagocytes from S100A9 deficient mice show altered migratory properties compared to wild-type cells. However, the underlying mechanisms are still unknown.
We established stable transfected HEK293 and NIH3T3 cell lines, which express S100A8/S100A9, S100A8, S100A9, S100A8/S100A9(N69A) (unable to form calcium induced tetramers) and S100A8/S100A9(T113A) (mutated phosphorylation site) and determined the individual contributions of the subunits and mutations to cellular dynamics. Using S100A8/S100A9 expressing HEK cells we also found a co-localization of the complex with microtubules comparable to what we have observed in monocytes.
Remarkable differences in S100A8/S100A9 transfected HEK cells compared to mock transfected ones were seen during breakdown and rebuilding of the microtubule (MT) based cytoskeleton indicating that S100A8/S100A9 affects the dynamic behaviour of MTs.
Cells from S100A9 deficient mice show altered characteristics in migration rates. Investigating this in more detail we found that S100A9 knockout cells are more adherent than wild-type cells. A similar pattern has also been observed for S100A8/S100A9 transfected HEK293 and NIH3T3 cells. These effects seem to be mediated by S100A8 but not by S100A9. Furthermore, the effects are only partially dependent on tetramerization of S100A8/S100A9 but require phosphorylation of S100A9.
Our main focus is now on transmigration studies using bone marrow cells of S100A9 wild-type and knockout mice. Our results provide evidence, that the S100A8/S100A9 complexes fulfil a pivotal role in remodelling cytoskeletal structures necessary for the migration of leukocytes.

P4.46

Influence of calcium ions on the conformation intrinsically disordered Starmaker protein

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Biomineralization is the process by which living organisms produce biominerals such bones, shells, teeth and otoliths. Such biominerals consist of two major components: calcium carbonate or phosphate which are the most abundant and proteins which play crucial role in proper formation of biominerals. Large number of these proteins are extremely acidic and highly phosphorylated and these features predispose them to bind alarge amount of calcium ions and controlling biominerals formation. Starmaker (Stm) is involved in biomineralization of otoliths in *Danio rerio*. Stm belongs to a class of intrinsically disordered proteins (IDPs) [1]. Stm demonstrates a low content of ordered secondary structure and no tightly packed hydrophobic core. Typical IDPs are very flexible, but may adopt rigid conformations in the presence of ligands or other proteins. Stm has a significantly extended rod-shaped conformation, however calcium ions, which are putative ligands of Stm, induce compaction of the extended conformation of Stm [2]. We have applied electron paramagnetic resonance (EPR) combine with site-directed spin labeling (SDSL) to examine the flexibility of Stm. Cysteine residues were introduced by site-directed mutagenesis and labeled with MSTL probe. The Stm conformational changes induced by calcium ions were detected by measurement of MSTL mobility. Phosphorylation of Stm by different kinases has been shown and the effect of phosphate groups on the Stm conformation has been analyzed.

References:

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Expression of ryanodine receptors in human trophoblast

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Trophoblasts of the placenta are the frontline cells involved in communication and exchange of materials between the mother and fetus. Within trophoblasts, Ca^{2+}-signalling proteins, such as Ca^{2+} channels, Ca^{2+} pumps and Ca^{2+} binding proteins are richly expressed (Moreau *et al.*, 2003). Intracellular free calcium ions are the key second messenger, participating in regulation of various cellular activities (Berridge *et al.*, 2003). In the placenta, transcellular Ca^{2+} transport through trophoblasts is also essential in formation of the fetal skeleton (Belkacmi *et al.*, 2005). Ryanodine receptors (RyRs) are high conductance cation channels that mediate Ca^{2+} release from intracellular Ca^{2+} stores such as the sarcoplasmic and endoplasmic reticulum to the cytoplasm. To date, the roles of RyRs in trophoblasts have not been reported. Studies from our laboratory revealed that RyRs are expressed in both human first trimester and term placental villous tissue within syncytiotrophoblast and cytotrophoblast cell layers. BeWo and JEG-3, model trophoblast cell lines were demonstrated to express functional RyRs in multiple isoforms. Changes in cytosolic free [Ca^{2+}] were observed in both BeWo and JEG-3 cells upon application of various RyR agonists and antagonists, using fura-2 fluorescent videomicroscopy. Furthermore, changes in cell volume in response to RyR agonists were also detected by using an intensiometric fluorophore calcein. These findings indicate that human trophoblasts express multiple RyR subtypes; pharmacological activation of RyRs in BeWo and JEG-3 cells elevates intracellular free [Ca^{2+}] level along with change of cell volume, suggest RyRs are potentially involved in osmoregulation in trophoblast.

References:

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Calcium dependence of TRPC4 and co-regulation by pertussis toxin-sensitive G proteins

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Transient receptor potential canonical 4 (TRPC4) and the closely related TRPC5 are calcium permeable non-selective cation channels involved in diverse cellular functions including neurotransmission, neurite outgrowth of the nervous tissues and neurogenic responses of smooth muscle cells in vascular and gastrointestinal systems. Gating of TRPC4 and TRPC5 usually involves transmitter-elicited activation of metabotropic receptors and consequent cell signaling through heterotrimeric G proteins. Although stimulation of phospholipase C *via* $G_{q/11}$-coupled receptors is sufficient to trigger activation of other TRPC channels, e.g. TRPC3, C6 and C7, the same maneuver does not always elicit a response in cells that express TRPC4 or TRPC5 in whole-cell voltage clamp experiments. More robust and/or complete activation of TRPC4 or TRPC5 channels is achieved with the co-stimulation of $G_{i/o}$-coupled and $G_{q/11}$-coupled receptors and the transducer functions appear to attribute mainly to the G-alpha subunits. With continued activation of $G_{i/o}$ proteins, a rise in intracellular Ca^{2+} concentration causes persistent activation of TRPC4. These results are consistent with the idea that TRPC4 and TRPC5 serve as coincidence detectors of the $G_{i/o}$ and $G_{q/11}$ signaling, with the latter being mainly supported through intracellular Ca^{2+} increase to trigger channel activation. Because the opening of these channels evoke robust membrane depolarization, the dependence on pertussis toxin-sensitive $G_{i/o}$ proteins suggests a novel excitatory function for of these "inhibitory G proteins" in neurotransmission and neuromuscular communication. Ca^{2+} influx through these channels in turn further potentiate the channel activity by a positive feedback mechanism to the extent that too high a Ca^{2+} concentration at the cytoplasmic side also causes channel inhibition.

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